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THE BIOSYNTHESIS AND METABOLISM OF HARMAN IN *PASSIFLORA EDULIS*—I.

THE BIOSYNTHESIS OF HARMAN

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Abstract—The β-carboline alkaloid harman has been shown by a combination of feeding and trapping experiments to be formed in *Passiflora edulis* from *N*-acetyltryptamine via harmalan. *N*-acetyltryptamine was shown to be formed from tryptophan via tryptamine. Tetrahydroharman is dehydrogenated to harman presumably via harmalan. Radioactive harman obtained from ^{14}C labelled *N*-acetyltryptamine, harmalan and tetrahydroharman was degraded to establish the position of the label and doubly labelled tryptophan was converted to *N*-acetyl tryptamine containing the same ration of $^{3}H:^{14}C$.

INTRODUCTION

ALKALOIDS based on the β-carboline ring system are amongst the simplest indole alkaloids and are distributed among a large number of genera. Altogether they have been reported in 14 genera, many in the family *Passifloraceae*.[1] There are 13 known alkaloids in the series made up of variations on five different oxidation states (Fig. 1); I (R = *R'* = H), I (R = OH, *R'* = H), II (R = OH, *R'* = H), III (R = *R'* = H) and III (R = OH, *R'* = H).

There is no direct experimental evidence for their biosynthesis, although Gröger and Simon[2] reported that β-^{14}C-tryptophan fed to *Peganum harmala* produced radioactive alkaloids. A number of biogenetic schemes have been proposed, the first of which was by Perkin and Robinson[3] who suggested that the skeleton of these molecules could be formed from tryptophan (after decarboxylation to tryptamine) and acetaldehyde. In support of this scheme it was shown[4] that tetrahydroharman (I, R = *R'* = H) can be readily synthesized from tryptamine hydrochloride and acetaldehyde in dilute aqueous solution at 25° using an acetate (pH 5·2) or phosphate (pH 6·2) buffer (Fig. 1).

A similar scheme has been proposed[5] for the biogenesis of a glycosidic alkaloid, the aglycone of which is harman-3-carboxylic acid (III, R = H, *R'* = COOH).

In this paper another biogenetic scheme is proposed (Fig. 2) for the biosynthesis of harman in *Passiflora edulis*. That is, that tryptophan is decarboxylated to tryptamine which after *N*-acetylation is cyclodehydrated to harmalan (V). Harmalan can then be oxidized to harman (VI) or reversibly reduced to tetrahydroharman. In support[6] of this hypothesis is an analogous series of reactions occurring in animal tissues, viz. the metabolism of melatonin (*N*-acetyl-5-methoxytryptamine) to 6-methoxyharmalan. Melatonin is formed from

[1] M. HESSE, In *Indolalkaloide in Tabellen*, p. 94, Springer-Verlag, Heidelberg (1964).
[2] D. GRÖGER and H. SIMON, *Abhandl. deut. Akad. Wiss. Berlin* 343 (1963).
[3] W. H. PERKIN JR. and R. ROBINSON, *J. Chem. Soc.* **115**, 933 (1919).
[4] G. HAHN and H. LUDEWIG, *Chem. Ber.* **67**, 2031 (1934).
[5] L. D. ANTONACCIO and H. BUDZIKIEWICZ, *Monatsh. Chem.* **93**, 962 (1962).
[6] S. KVEDER and W. M. MCISAAC, *J. Biol. Chem.* **236**, 3214 (1961).

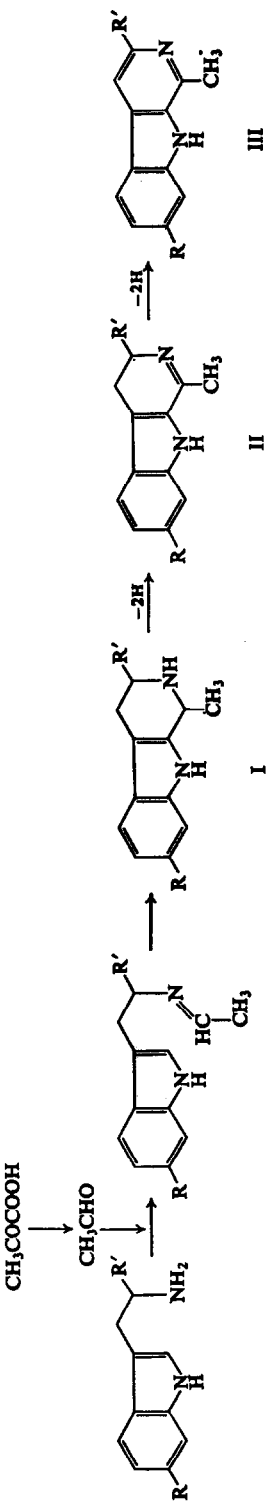
CH3COCOOH
CH3CHO
R'
NH2
R
N
H
HC
CH3
NH
-2H
I
II
III
CH3
N

FIG. 1.

COOH
NH2
CO2
CH3CO—SCoA
NH
CO
CH3
H2O
-2H
+2H
IV
V
VI
CH3
N
H

FIG. 2.

5-hydroxytryptamine by *N*-acetylation followed by *O*-methylation and is converted, presumably by cyclodehydration, to 6-methoxyharmalan.

RESULTS AND DISCUSSION

Of all the plants in which the β-carboline alkaloids occur the one most readily accessible and easy to grow under Sydney conditions is *Passiflora edulis*, the common passion-fruit, although it contains a low concentration (4 μg/g fresh wt)[7] of harman only in the stem and leaves. For this reason *in vivo* feeding experiments were carried out using vigorously growing shoots from mature plants and particularly two leaf seedlings both etiolated and normal.

The initial step in the biosynthesis of alkaloids derived from amino acids appears to be a decarboxylation because of the efficient incorporation of both the amino acid and its decarboxylation product into the alkaloids, although the only amino acid decarboxylase which has been purified from higher plants is glutamic acid decarboxylase.[8] Exceptions among the indole alkaloids are harman-3-carboxylic acid which is presumably formed by initial acetylation of tryptophan, and cordifoline[9] which is a substituted harman-3-carboxylic acid. For this reason *N*-2-^{14}C-acetyl-tryptophan was fed to *P. edulis* shoots. It showed no detectable incorporation into either harmalan or harman, suggesting that the first step in the biosynthesis of harman in *P. edulis* is the decarboxylation of tryptophan to tryptamine. In support of this suggestion is the presence of tryptamine (40 μg/g fresh wt) in the plant.[7]

The results obtained (Tables 1, 2, 3 and 4) from feeding and trapping experiments support the proposal that *N*-acetyltryptamine is derived from tryptophan via tryptamine presumably by *N*-acetylation of tryptamine by acetyl CoA. The β-carboline ring is then formed by cyclic dehydration of *N*-acetyltryptamine (IV) to give harmalan (V) and this is dehydrogenated to harman (VI) as shown in Fig. 2.

TABLE 1. FEEDING EXPERIMENTS: CONVERSION OF POTENTIAL PRECURSORS TO HARMAN

Substrate	μmoles absorbed	μmoles of ^{14}C-harman formed
N-2-^{14}C-acetyltryptophan	1·540	0·000
β-^{14}C-tryptophan	$1{\cdot}8 \times 10^{-3}$	$2{\cdot}2 \times 10^{-6}$
β-^{14}C-tryptamine hydrochloride	0·105	$1{\cdot}8 \times 10^{-3}$
N-2-^{14}C-acetyltryptamine*	0·678	0·015
^{14}C-tetrahydroharman hydrochloride†	1·332	0·084
^{14}C-harmalan hydrochloride	0·359	0·036

All feeding experiments were carried out at least 3 times and values quoted represent individual experiments.

* Etiolated seedlings were used.

† Rapidly growing shoots were used. 0·150 μmoles of harmalan were also formed.

[7] M. SLAYTOR and C. R. DEAN, unpublished results.
[8] O. SCHALES, V. MIMS and S. S. SCHALES, *Arch. Biochem. Biophys.* **10**, 455 (1946).
[9] R. T. BROWN and L. R. ROW, *Chem. Commun.* 453 (1967).

The ^{14}C and ^{3}H labelled compounds which were used for the feeding and trapping experiments were either synthesized or were commercially available. *N*-Acetyltryptamine could not be detected in the plant but it was possible to show that it is formed in the plant by trapping experiments (Table 2) in which unlabelled *N*-acetyltryptamine was fed to *P. edulis* followed 24 hr later by either labelled tryptophan or tryptamine. The *N*-acetyltryptamine isolated 3 days later after feeding β-^{14}C-tryptamine was radioactive.

TABLE 2. TRAPPING EXPERIMENTS

Unlabelled trap	μmoles added	Radioactive substrate	μmoles absorbed	μmoles of radioactive substrate converted
N-acetyltryptamine	2·6	β-^{14}C-tryptamine	0·069	$7{\cdot}1 \times 10^{-3}$*
Harmalan hydrochloride	0·051	β-^{14}C-tryptophan	$1{\cdot}68 \times 10^{-3}$	0·00

Trapping experiments were carried out at least twice and values quoted represent individual experiments.

* This was hydrolysed to tryptamine of the same specific activity.

It was not possible to degrade the *N*-acetyltryptamine isolated after feeding β-^{14}C-tryptamine. It was hoped to do this by hydrolysis to tryptamine and a Hofmann elimination on the quaternary hydroxide formed by reaction with methyl iodide followed by cleavage of the vinyl group to ^{14}C-formaldehyde. The ^{14}C-*N*-acetyltryptamine was hydrolysed to ^{14}C-tryptamine of the same specific activity but the 3-vinylindole could not be prevented from polymerizing. The polymerization of 3-vinylindole has been noted previously.[10] In a similar experiment doubly labelled L-tryptophan (^{3}H-(G) and β-^{14}C) was fed and, because of quenching of the tritium, the *N*-acetyltryptamine trapped was burnt to $^{3}H_2O$ and $^{14}CO_2$. The ratio of ^{3}H:^{14}C in the *N*-acetyltryptamine was found to be slightly lower than that of the tryptophan fed presumably due to loss of the α-H of tryptophan during decarboxylation (Table 3). This result, together with the trapping experiment with β-^{14}C-tryptamine indicate the sequence of reactions tryptophan to tryptamine to *N*-acetyltryptamine.

TABLE 3. CONVERSION OF ^{3}H:^{14}C-L-TRYPTOPHAN TO ^{3}H:^{14}C-*N*-ACETYLTRYPTAMINE

μmoles of *N*-acetyl tryptamine added	Radioactive L-tryptophan added	Counts/min	^{3}H:^{14}C ratio	^{3}H:^{14}C-*N*-acetyltryptamine $^{3}H(as\,^{3}H_2O)$ counts/min	$^{14}C(as\,^{14}CO_2)$ counts/min	Ratio
0·13	^{3}H-(G) ^{14}C-β	$5{\cdot}6 \times 10^{4}$ $2{\cdot}6 \times 10^{4}$	2·14:1	122	60	2·03:1

Harman isolated from the plant after feeding experiments with ^{14}C labelled precursors was expected to be specifically labelled. The methyl group could be easily isolated from harman by Kuhn–Roth oxidation. Harman isolated from feeding experiments with labelled *N*-acetyltryptamine, harmalan and tetrahydroharman was degraded to acetic acid which

[10] B. WITKOP, *J. Am. Chem. Soc.* 71, 2559 (1949).

contained the expected amount of radioactivity (Table 4). Acetic acid from Kuhn–Roth oxidation of harman formed from *N*-2-^{14}C-acetyltryptamine was degraded to methylamine of the same specific activity as the harman. The results in Tables 1 and 4 show that *N*-acetyltryptamine is converted to harman as shown in Fig. 2.

TABLE 4. KHUN–ROTH OXIDATIONS OF HARMAN

Precursor	Specific activity (counts/min/mmole)	Amount* (mg)	CH_3COOAg (% of harman activity)	$CH_3NH_2.HCl$ (% of harman activity)
N-2-^{14}C-acetyltryptamine†	841	17·7	89	—
N-2-^{14}C-acetyltryptamine	127	57·2	—	97
^{14}C-tetrahydroharman hydrochloride	379	25·3	125	—
^{14}C-harmalan hydrochloride‡	706	21·0	102	—

* This was further diluted to 50 mg for the Khun–Roth oxidation.
† Etiolated seedlings were used for the feeding experiment.
‡ A crude homogenate was used for this experiment.

Harmalan cannot be detected in *P. edulis* and, although it is converted to harman (Table 1), it could not be conclusively shown that it is an intermediate in the biosynthesis of harman because it could not be trapped (Table 2). Possibly the presence of large amounts of harmalan present as the trap could produce enough harman to prevent any further alkaloid biosynthesis from taking place. It has been noted[7] that feeding large amounts of harman to *P. edulis* results in greatly increased levels of tryptophan, presumably due to the same type of control.

The essential difference between Robinson's scheme (Fig. 1) and that proposed here (Fig. 2) is that in the latter the first tricyclic intermediate is harmalan and not tetrahydroharman. However tetrahydroharman is converted to harmalan and thence to harman in *P. edulis* (Table 1) suggesting an *in vivo* system involving two successive dehydrogenations. Tetrahydroharman cannot be detected in *P. edulis* though it is much more difficult to detect than harman. Tetrahydro-β-carboline alkaloids occur in a number of plants including *Banisteria caapi* which also produce a dihydro-β-carboline and a β-carboline alkaloid.[1] If the pathway established here for the biosynthesis of harman in *P. edulis* is the only one operating for the biosynthesis of β-carboline alkaloids, then the tetrahydro-β-carbolines could arise by reduction of dihydro-β-carbolines, i.e. the conversion of tetrahydroharman to harmalan in *P. edulis* would be reversible. The results presented here do not exclude the existence of the alternative pathway (Fig. 1).

This work is being extended to a study of the enzymes involved. Preliminary experiments with a crude homogenate have demonstrated the conversion of harmalan to harman (Table 4).

EXPERIMENTAL

Materials and Methods

L-β-^{14}C-Tryptophan (specific activity 32 mCi/mmole) and L-^{3}H-(G)-tryptophan (specific activity 500 mCi/mmole) were purchased from the Radiochemical Centre, Amersham.

Thin layer chromatography (TLC) plates using Silica Gel G were prepared according to Chalmers.[11] The developing solvent was methanol. The alkaloids were identified by their fluorescence under u.v. light or their colour with iodine.

[11] A. H. CHALMERS, C. C. J. CULVENOR and L. W. SMITH, *J. Chromatogr.* **20**, 270 (1965).

^{14}C and ^{3}H were counted on Packard Tri-carb Liquid Scintillation Spectrometers (Models 500B or 3375). 15 ml of scintillant (containing 4 g of PPO and 100 mg POPOP/l of toluene) and 1 ml of methanol was used per counting vial. The TLC scrapings and silver acetate were suspended in scintillant containing 500 mg of Packard thixotropic gel powder.

Ultra-violet spectra were run using a Cary Model 14 recording spectrophotometer. A Köfler hot stage-microscope was used for determining melting points which are uncorrected.

Feeding Experiments

All plant material was grown in a temperature controlled greenhouse at 70°F.

Three types of plant material from *P. edulis* (Norfolk Island Strain) were used: 1. Growing shoots from mature plants; 2. Etiolated seedlings; and 3. Growing seedlings. These were obtained by germinating seeds in sand, and were used at the two leaf stage (11–13 days after germination). Unless otherwise stated, all feeding and trapping experiments were carried out using shoots from these seedlings.

Each of the two types of seedlings (in groups of three) and the shoots from mature plants, cut above the roots under water, were placed in a 1 ml beaker containing the substrate dissolved in water (0·5–0·8 ml) and allowed to grow for 3 days, extra water being added as required.

In the trapping experiments the unlabelled trap was added 24 hr before the radioactive substrate and the shoots were then allowed to grow for 3 days.

In vitro Conversion of Harmalan to Harman

^{14}C-harmalan (0·305 μmoles) was incubated for 4 hr at 37° with a crude homogenate (equivalent to 19 g of growing seedlings) prepared from an acetone powder[12] homogenized with phosphate buffer (0·1 M; pH 7·0) and centrifuged at $700 \times g$. The enzyme reaction was stopped by addition of alkali and carrier harman then added. The alkaloids were extracted and separated by TLC. Appropriate controls were carried out. Net conversion to harman, 0·021 μmoles.

Isolation of Alkaloids

The plant was ground in a Ten Broeck homogenizer with 75 per cent ethanol and the filtered extract washed through a Zeocarb-225 column in the acid form, made up in 75 per cent ethanol. *N*-2-^{14}C-acetyltryptamine was isolated after the addition of carrier by vacuum sublimation of the unexchanged material. The *N*-acetyltryptamine was sublimed to constant specific activity. The basic fraction was eluted with 75 per cent ethanol made 3 *N* with respect to ammonia, and after adding 50 μg of appropriate carrier was separated by TLC and then eluted with methanol. The alkaloid HCl-ide was recrystallized to constant specific activity after addition of 100 mg of unlabelled material from methanol:ether (1:5). Alternatively the basic fraction was diluted with 80 mg of harman, vacuum sublimed once and twice recrystallized from methanol:ether as the HCl-ide.

Kuhn–Roth Oxidation

This was carried out according to Cornforth.[13]

Schmidt Decarboxylation

This was carried out according to Phares.[14]

Combustion of ^{14}C-^{3}H-N-acetyltryptamine

This was carried out by strongly heating for 2 min an intimately ground mixture of ^{14}C-^{3}H-*N*-acetyltryptamine (3·4 mg) and CuO (5 mg) in a stream of O_2. The $^{3}H_2O$ was collected in a cold trap (ice-salt) and the $^{14}CO_2$ absorbed in hyamine hydroxide.

Chemical Syntheses

N-2-^{14}C-*acetyltryptamine.* Acetic-2-^{14}C anhydride (0·10 mC; 5·1 mg) was transferred by heating under vacuum to a solution of acetic anhydride (0·22 ml), tryptamine (178 mg) and sodium acetate (2·7 mg) frozen in liquid air. The reaction mixture was then heated on a water bath for 2 hr when the excess acetic anhydride was removed under vacuum. *N*-2-^{14}C-acetyltryptamine was recrystallized from ether:light petroleum (b.p. 60–90°). Yield: 187 mg, (84 per cent); specific activity 16·4 μCi/mmole; single spot on TLC (developed with Ehrlich's reagent).

[12] A. NASON, In *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. I, p. 62, Academic Press, New York (1955).
[13] J. W. CORNFORTH, R. H. CORNFORTH, A. PELTER, M. G. HORNING and G. POPJAK, *Tetrahedron* **5**, 311 (1959).
[14] E. F. PHARES, *Arch. Biochem. Biophys.* **33**, 173 (1951).

1-^{14}C-*Methyl-3,4-dihydro-β-carboline hydrochloride* (*harmalan hydrochloride*). This was synthesized according to Späth and Lederer.[15] From *N*-^{14}C-acetyltryptamine (176 mg), 103 mg (54 per cent) of harmalan HCl-ide was obtained, m.p. 243–5°; specific activity 14·3 μCi/mmole. The harmalan for *in vivo* and *in vitro* experiments was purified by separation from traces of harman and other impurities by TLC.

1-^{14}C-*methyl*-1,2,3,4-*tetrahydro-β-carboline* (*tetrahydroharman*) *hydrochloride*. ^{14}C-Harmalan hydrochloride (51 mg) dissolved in a suspension of PtO_2 (19·7 mg) in acetic acid (15 ml) was shaken under H_2 at 350 psi for 3 hr. After filtering off the catalyst, the acetic acid was removed under red. pressure at 100°. The residue was dissolved in a small volume of methanol:water, basified with NaOH and extracted 3× with benzene. The benzene solution was dried (Na_2CO_3) and evaporated to dryness to give a pale brown solid. This was dissolved in methanol containing conc HCl, and the tetrahydroharman HCl crystallized from methanol:ether as straw coloured crystals. Yield: 35·2 mg (68 per cent); ϵ_{227} 35,200 (reported value[16] ϵ_{225} 32,100); specific activity 6·65 μCi/mmole. The tetrahydroharman for feeding experiments was separated from contaminating harmalan and harman by TLC.

N-2-^{14}C-*Acetyltryptophan*. This was synthesized according to Warnell and Berg.[17] L-Tryptophan (204 mg) was dissolved in 0·5 *N* NaOH (2 ml) and 2-^{14}C-acetic anhydride (102 mg) added. The mixture was allowed to stand for 2 hr at room temperature and then acidified with conc HCl. The resulting gum crystallized slowly from aqueous ethanol containing a trace of HCl. Yield: 132 mg (54 per cent); m.p. 201–3° (reported[17] 205°); specific activity 5·13 μCi/mmole.

β-^{14}C-*Tryptamine*. Dimethyl sulphate (0·06 ml), Na^{14}CN (0·5 mCi; 0·68 mg) and KCN (38·5 mg) were added to a vigorously shaken solution of gramine (91·8 mg) in aqueous dioxan (2·5 ml; 20 per cent) over 8 min. After standing for 3 hr the indole acetonitrile was separated from unchanged gramine and obtained as an oil which was not further purified. Raney Ni and NaOH (2*N*) were added over 2 hr to a stirred solution of the indole acetonitrile in aqueous ethanol (8 ml) and NaOH (0·6 ml; 2*N*) at room temperature. Tryptamine was recovered from the filtrate after separation from unchanged starting material and purified on preparative TLC. Yield of β-^{14}C-tryptamine HCl-ide 4·2 mg; specific activity, 0·315 mCi/mmole.

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[15] E. Späth and E. Lederer, *Chem. Ber.* 63, 120 (1930).
[16] N. Neuss, In *Physical Data of Indole and Dihydroindole Alkaloids*, Vol. 1, p. 121, Eli Lilly, Indiana (1964).
[17] J. L. Warnell and C. P. Berg, *J. Am. Chem. Soc.* 76, 1708 (1954).